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(57) Abstract

Live *Brucella* vaccines and methods for preparing the live vaccines protective against brucellosis are described. The vaccines are prepared by introducing a deletion in the *rfbU* gene of a strain of *Brucella* which results in attenuation of the strain while retaining the desired immunogenicity to initiate a protective immunogenic response. Other strains with varying levels of attenuation are described.

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TITLE OF THE INVENTION

Live Vaccine Against Brucellosis

INTRODUCTION

Brucella infects a significant number of people and livestock in developing countries and infects wild as well as domestic animals in the United States. In addition, *Brucella* is a potential biowarfare agent; strains of *Brucella* have been constructed with resistance to multiple antibiotics used to treat the disease. These strains pose a significant morbidity and mortality threat to exposed personnel. Brucellosis symptoms include recurring fever, chills and anxiety. Even though the disease is rarely fatal, once well established, the disease is difficult to treat since the bacteria reside in the bone marrow.

Two live attenuated *Brucella* strains currently approved for use as animal vaccines, *B. abortus* Strain 19 [Cheville, et al. (1993) *Am. J. Vet. Res.* **54**: 1591-1597; Brucellosis research: an evaluation. Report of the subcommittee on Brucellosis Research, National Academy of Sciences. Washington, D.C.: National Academy Press, 1977: 61-77] and *B. melitensis* strain

Rev 1 [Jimenez de Bagues, M. P. et al. (1989) *Ann. Rech. Vet.* **20**:205-213; Pardon, P. et al. (1990) *Ann. Rech. Vet.* **21**:153-160], are not ideal vaccine strains. Both strains cause vaccinated animals to seroconvert and thus make subsequent serological diagnosis of brucellosis difficult [Jimenez de Bagues, M. P. et al. (1992) *Vet. Microbiol.* **30**:233-241]. Both strains can induce abortion (Jimenez de Bagues, 1989, *supra*; Corner, L. A. and Alton G. G. (1981) *Res. Vet. Sci.* **31**:342-344] and both can cause disease in humans [Blasco, J. M. and R. Diaz (1993) *Lancet* **342**:805; Young, E. J. (1983) *Rev. Inf. Dis.* 5: 821-842]. A more recent attenuated strain of *B. abortus*, RB51 [Schurig, G. G. et al. (1991) *Vet. Microbiol.* **28**: 171-188], shows more promise as a live vaccine strain. RB51 is a rough strain that confers protection against infection by *Brucella*, yet does not cause seroconversion [Cheville, N. F. 1993, supra; Jimenez de Bagues, M. P. et al. (1994) *Infect. Immun.* **62**: 4990-4996]. However, neither the genetic basis of the RB51 rough mutation nor the basis of attenuation is known. Also, RB51 carries resistance to rifampin, an antibiotic currently used to treat brucellosis.

Therefore, there is a need for a live attenuated *Brucella* vaccine strain, with a defined nonreverting genetic mutation, which does not cause seroconversion in the vaccinee, and which does not retain resistance to antibiotics used in the treatment of brucellosis.

SUMMARY OF THE INVENTION

The present invention fulfills the need described above. In this application is described attenuated rough strains of *Brucella*, containing genetically defined mutations, which will not cause seroconversion. The mutations in these attenuated

Brucella strains were created by DNA deletion, the type of mutation least susceptible to genetic reversion and are therefore advantageous as vaccine strains. These vaccines strains do not retain resistance to an antibiotic useful for treatment of brucellosis.

More specifically, this invention relates to two genetically defined rough mutants of *Brucella melitensis*, WRR51 and WRRP1, as candidate strains for a live vaccine against brucellosis. These strains differ from *Brucella* live vaccines currently used in livestock because they have genetically defined mutations that were created by deleting DNA from the *Brucella* chromosome. Both strains have a lipopolysaccharide (LPS) defect and thus do not cause the seroconversion that complicates disease screening. Smooth strains currently approved for use in animals are not good candidates for human vaccines because though attenuated, they can still cause disease in humans. One of the vaccine strains of the present invention, WRRP1, is a double deletion mutant that is highly attenuated and is unlikely to cause disease in humans.

Briefly, the genetically defined rough mutants of *Brucella* were constructed by using a *Brucella abortus* VTRA1 chromosome containing a Tn*5* insertion which conferred a rough phenotype [Winter, A. J. et al. (1996) *Amer. J. Vet. Res.* **57**: 677-683]. The *B. abortus* gene containing the Tn*5* insertion was cloned from the VTRA1 chromosome and the nucleotide sequence of the 2693 bp (SEQ ID NO:1) region containing the transposon insertion was determined. The Tn*5* insertion was found to be located within an open reading frame of 1233 bp spanning nucleotides 883 through 2115 of SEQ ID NO:1 which coded for a gene that was distantly related (40%

amino acid similarity) to the sequence of the *Salmonella enterica LT2 rfbU*, a gene encoding a mannosyltransferase [Liu, D. et al. (1993) *J. Bacteriol.* **175**: 3408-2414]. A deletion of 607 bp was made in the putative *rfbU* gene and a cassette containing a chloramphenicol acetyl transferase gene (*cat*) was ligated into the deletion site to create *rfbU/cat*. The plasmid containing *rfbU/cat*, pRFBU1, was electroporated into *B. melitensis* strain 16M and electroporants with pRFBU1 integrated were selected on *Brucella* agar containing chloramphenicol. Southern DNA hybridization confirmed that the chloramphenicol resistant and ampicillin sensitive electroporants had the deletion mutation carrying the chloramphenicol resistance cassette in place of the wild type chromosomal locus resulting from a directed allelic exchange by a double crossover recombinational event. The deletion strain, designated WRR51, was confirmed to be rough by staining with crystal violet, and by lack of agglutination with an anti-LPS serum.

A *purE* deletion was then introduced into *B. melitensis* strain WRR51 by a similar allelic exchange procedure. PurE is an essential enzyme in the purine biosynthetic pathway. The resultant double deletion strain (Δ*rfbU* Δ*purE*) was designated WRRP1. The DNA flanking the transposon insertion was sequenced to determine the open reading frame that had been interrupted to cause the rough phenotype and was found to be *rfbU*. The complete sequence of *Brucella rfbU* is described for the first time in this application in SEQ ID NO:1.

Unlike the rough mutants of the present invention, none of the rough mutants described previously including *B. abortus* strain 2308 *rfbU* mutant, VTRA1, and the VTRA1 transposon mutation

integrated into the chromosomes of *B. melitensis* and *Brucella suis* by allelic exchange to create VTRM1 and VTRS1, respectively [McQuiston, J. R. et al. (1995) Abstract, CRWAD, Nov. 1995; Winter, A. J. et al. (1996) *Am. J. Vet. Res.* **57**:677-683] contained a defined mutation. In other words, the previously described mutant strains were produced by a transposon insertion which is a random event and can occur at any chromosomal location wherein the mutants of the present invention were produced by a directed allelic exchange to produce a unrevertable, defined deletion in the gene. A plasmid construct containing a synthetic copy of the putative *rfbU* gene that restored the smooth phenotype to the WRR51 deletion mutant of the present invention, did not restore the smooth phenotype to the VTRA1 transposon mutant. The inability to complement the transposon mutant indicates either that the transposon insertion confers a more general genetic defect in LPS biosynthesis (via a polar effect), or that the VTRA1 strain has additional mutations that affect LPS biosynthesis. The rough mutants of the present invention have a defined, nonreverting, deletion in the putative *rfbU* gene that was integrated into the chromosome by allelic exchange.

In order to construct the deletion in a rough strain, several factors had to be considered. The sequence of the flanking DNA (the *rfbU* gene) extending far enough in either direction of the deletion had to be known to allow for PCR or direct cloning of a large enough region of the *Brucella* chromosome. In addition, it was important to allow for a deletion of a significant portion of the *rfbU* gene to inactivate the gene in the first attempt; the actual crossover (allelic exchange) of the Δ*rfbU* for the wild type was

very difficult because it occurred at a very low frequency, and after several trials, it was found that a threshold of at least 500 bp on either side of the deletion was necessary for efficient homologous recombination crossover in the *Brucella* chromosome. High biocontainment facilities, Biosafety Level 3 (BSL3), were necessary to move the deletion construct back into *Brucella* to make the mutant. Introducing the deletion construct required development of a more efficient method for electroporating DNA into *Brucella* than used before.

Therefore, it is an object of the present invention to provide a *rfbU* DNA fragment encoding 2693 nucleotides useful as a diagnostic agent.

It is another object of the present invention to provide an amino acid sequence for RfbU protein encoding 411 amino acids.

It is another object of the present invention to provide a *Brucella rfbU* DNA fragment containing a deletion useful in attenuating a *Brucella* strain.

It is another object of the invention to provide a recombinant vector comprising a vector and any of the above described DNA fragments.

It is a further object of the present invention to provide a host cell transformed with any of the above-described recombinant DNA constructs.

It is another object of the present invention to provide a method for producing RfbU protein which comprises culturing a host cell under conditions such that a recombinant vector comprising a vector and the *rfbU* DNA fragment is expressed and RfbU protein is

thereby produced, and isolating RfbU protein for use as a diagnostic agent.

It is a further object of the present invention to provide an antibody to the above-described RfbU for use as a diagnostic agent.

It is yet another object of the invention to provide a *Brucella spp.* vaccine comprising an attenuated rough *Brucella* containing a defined deletion in the *rfbU* gene and effective for the production of antigenic and immunogenic response resulting in the protection of an animal against brucellosis. All of the *Brucella* which infect humans are highly related, probably biovars of the same species [Corbel, M. J. (1997) Emerging Inf. Dis. 3:213-221]. It is expected that this live vaccine would provide cross protection against other *Brucella* strains since there is thought to be high homology in the *rfbU* gene in brucellae [Jimenez de Bagues, M. P. et al. (1994) Infect. and Immun. 62: 4990-4996].

It is a further object of the invention to provide a multivalent *Brucella* vaccine comprising defined *Brucella rfbU* mutants from a variety of strains effective for the production of antigenic and immunogenic response resulting in the protection of an animal against infection with brucellae.

It is yet another object of the present invention to provide a method for the diagnosis of brucellae infection comprising the steps of:

(i) contacting a sample from an individual suspected of having the infection with antibodies which recognize RfbU protein; and

(ii) detecting the presence or absence of a complex formed between RfbU and antibodies specific therefor.

It is yet another object of the present invention to provide a method for the diagnosis of *Brucella* in a sample using the polymerase chain reaction, said method comprising:

(i) extracting DNA from the sample;

(ii) contacting said DNA with

(a) at least four nucleotide triphosphates,

(b) a primer that hybridizes to *rfbU* DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

(iii) denaturing said duplex to release said first DNA product from said DNA;

(iv) contacting said first DNA product with a reaction mixture comprising:

(a) at least four nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

(v) denaturing said second DNA product from said first DNA product;

(vi) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

(vii) fractionating said first and second DNA products generated from said *rfbU* DNA; and

(viii) detecting said fractionated products for the presence or absence of *rfbU* in a sample.

It is yet another object of the present invention to provide a method for the detection of *Brucella spp.* in a sample which comprises assaying for the presence or absence of *rfbU* RNA or DNA in a sample by hybridization assays.

It is a further object of the present invention to provide a diagnostic kit comprising a RfbU antibody and ancillary reagents suitable for use in detecting the presence of brucellae in mammalian tissue or serum.

It is a further object of the present invention to provide a diagnostic kit comprising primers specific for the amplification of *rfbU* sequences and ancillary reagents suitable for use in detecting the presence of brucellae in mammalian tissue or serum.

It is yet an object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of brucellosis, said method comprising providing to an individual in need of such treatment an effective amount of sera from individuals immunized with the vaccine strains of the present invention in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a means to express antigens of interest as potential therapeutics or vaccines for human and veterinary use. RfbU is usually either cytoplasmic or associated with the inner membrane. When brucellae are lysed within host cells, RfbU and any antigen designed to be expressed with RfbU would then be accessible to the intracellular environment of the cell or host.

It is another object of the invention to provide an inactivated vaccine produced from the live attenuated *Brucella* described above. The attenuated *Brucella* of the present invention can be used in producing inactivated *Brucella* vaccines. By using an attenuated *Brucella*, particularly the double mutant which is significantly attenuated, there is a much greater margin of safety in the event that the product is incompletely inactivated. Starting with an attenuated strain is also much safer during the manufacturing phase, and may allow production under lower biocontainment levels. In addition, inactivated attenuated *Brucella* strains can be used to isolate subunits for subunit vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1 shows ability of rough mutants to grow within human host cells.

Figure 2 shows the ability of rough mutants to infect mice via the intraperitoneal route.

DETAILED DESCRIPTION

In one embodiment, the present invention relates to a DNA or cDNA segment which encodes RfbU, a mannosyltransferase. The sequence of the gene, specified in SEQ ID NO: 1, was obtained by cloning out a *Brucella abortus* VTRA1 chromosome containing a Tn5 transposon and sequencing the subclones to determine the insertion site of Tn5 in the VTRA1 chromosome.

The sequenced gene fragment comprising 2693 base pairs contains an open reading frame of 1233 base pairs.

DNA or polynucleotide sequences to which the invention also relates include sequences of at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 nucleotides, most preferably at least about 15-20 nucleotides corresponding, i.e., homologous to or complementary to, a region of the *rfbU* nucleotide sequence. Preferably, the sequence of the region from which the polynucleotide is derived is homologous to or complementary to a sequence which is unique to the *rfbU* gene. Whether or not a sequence is unique to the *rfbU* gene can be determined by techniques known to those of skill in the art. For example, the sequence can be compared to sequences in databanks, e.g., GenBank and compared by DNA:DNA hybridization. Regions from which typical DNA sequences may be derived include but are not limited to, for example, regions encoding specific epitopes, as well as non-transcribed and/or non-translated regions.

DNA sequences also embodied in the present invention include the *rfbU* sequence containing a deletion. The exemplified deletion described below in the Examples is 607 base pairs in length, spanning from position 1063 to position 1670 of the DNA sequence identified in SEQ. ID. NO:1 and was chosen because of conveniently located restriction sites. Other deletions of any size can be introduced into the *rfbU* gene for different purposes. In the case where RfbU function is to be eliminated, a deletion large enough to eliminate any possibility of recombinational restoration of gene function is preferable. The deletion need not be framed by restriction sites and can be introduced by PCR, for example. However,

cloned homologous DNA of about 500 base pairs flanking the deletion site is necessary for efficient allelic replacement. Methods for manupulating genes in *Brucella* are known in the art, please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning: a Laboratory Manual (1982) or DNA Cloning, volumes I and II (D. N. glover ed. 1985) or Current Protocols in Molecular Biology, Ausubel, F. M. et al. (Eds.) John Wiley & Sons, Inc., for general cloning methods.

The derived polynucleotide is not necessarily physically derived from the nucleotide sequence shown in SEQ ID NO:1, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain reaction assays and for the discovery of other *rfbU* sequences.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid, such as pUC19, or any other vector which replicates in *E. coli*, or a suicide vector, or broad host range vectors for example pTh10(IncP), pSa(IncW) and R751(IncP) [Rigby, C. E. and A.D.E. Fraser (1989) *Can. J. Vet. Res.* **53**:326-330] and others known in the art.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be any *Brucella* or other host cell for which the vector is designed. The vector containing the *rfbU* gene is expressed in the bacteria and the product can be isolated for use in diagnostic assays. For example, the plasmid pRFBU1, described below in Materials and Methods, containing the *rfbU/cat* construct can be electroporated into other brucellae, and by allelic exchange with the wild type form of *rfbU,* can attenuate the electroporants. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of RfbU protein. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to a RfbU protein having an amino acid sequence corresponding to SEQ ID NO: 2 and encompassing 411 amino acids or any allelic variation thereof.

A polypeptide or amino acid sequence derived from the amino acid sequence in SEQ ID NO:2, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at

least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence, or the sequence in SEQ ID NO:1; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides for example, any protective antigen of any pathogen, bacterial or viral for secretion of heterologous antigens from within the host cell since RfbU is either cytoplasmic or associated with the inner membrane. In addition, the protein or polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, thereby producing higher titers of neutralizing antibody when used as a vaccine. Examples of such proteins or polypeptides include any adjuvants or carriers safe for human use, such as aluminum hydroxide.

In another embodiment, the present invention relates to antibodies specific for the above-described RfbU protein. For instance, an antibody can be raised against the complete RfbU protein or against a portion thereof. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the polypeptide of the present invention. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986).

In a further embodiment, the present invention relates to a method for detecting the presence of brucellosis infection or antibodies against *Brucella* in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of the RfbU protein, or alternatively, inactivated attenuated *Brucella* described above, and contacting it with the serum of a person suspected of having a brucellosis infection. The presence of a resulting complex formed between the antigen (RfbU or attenuated *Brucella*) and antibodies specific therefor in the serum can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis and typing of brucellosis infections.

In yet another embodiment, the present invention relates to a method of detecting the presence of *Brucella* in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), antibodies specific for RfbU, and contacting it with serum or tissue sample of a person suspected of having a brucellosis infection. The presence or absence of a resulting complex formed between RfbU in the serum or presented on antigen presenting cells and antibodies specific therefor can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis

of a brucellosis infection or for typing the specific *Brucella* bacteria causing such an infection.

In another embodiment, the present invention relates to a diagnostic kit which contains RfbU from a specific strain or species of *Brucella* or several different strains and species of *Brucella* and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence of antibodies to *Brucella* in serum or a tissue sample. Tissue, blood, serum, or urine samples contemplated can be animal, in particular abortion products of large animals, or human, or other vertebrates. Other samples contemplated include dairy products, especially unpasturized products, from which the disease is most frequently contracted.

In yet a further embodiment, the present invention relates to DNA or nucleotide sequences for use in detecting the presence or absence of *Brucella* using the polymerase chain reaction (PCR). Since *rfbU* is probably very similar at the DNA level across all *Brucella*, the DNA sequence of the present invention can be used to design primers which specifically bind to the *rfbU* DNA for the purpose of detecting the presence, absence, or quantitating the amount of *Brucella*. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides. Reagents and controls necessary for PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of *rfbU* sequences, for example by gel fractionation, with or without hyridization, by radiochemistry, and immunochemical techniques. This method can also be used for typing a brucellosis infection.

In yet another embodiment, the present invention relates to a diagnostic kit which contains PCR primers specific for *rfbU*, and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence or absence of *Brucella* in a sample using PCR. Samples contemplated can be human or other mammals, dairy products, blood, urine, tissues, depending on the stage of infection. Early infection tissues would include lungs and the lymphatic system, late infection tissues would include spleen, liver, and kidneys, and later infection tissues would include bone marrow and absesses in organs and brain.

In another embodiment, the present invention can be used to diagnose *Brucella* infection by using the DNA sequences for detecting the presence or absence of *rfbU* in a DNA sample using hybridization assays such as Southern hybridization or the expression of *rfbU* gene, or *rfbU* RNA, by northern hybridizations and other hybridization assays well known to a person with ordinary skill in the art.

In another embodiment, the present invention relates to a vaccine for protection against infections by *Brucella*. The vaccine comprises one or more attenuated rough *Brucella* strains containing a defined deletion, for example, in the *rfbU* gene. The deletion in the *rfbU* gene can be introduced by allelic exchange due to a double cross-over recombinational event, or any other method wherein a DNA replacement event in which two separate DNA recombination events result in the exchange of a piece of the intact gene for a homologous piece containing a deletion. The deletion is preferably large enough such that the gene is inactivated in the first attempt and to reduce the likelihood of a recombinational repair. Other genes

which can be deleted include *purE*, *dnaKJ*, *recA*, *groELS*, *catalase*, or any other gene which contributes to survival in human macrophages and/or to bacterial virulence. Any strain of *Brucella* can be used to introduce such an attenuating mutation. The resulting attenuated strain can be tested for the deletion of the targetted gene by methods known in the art such as Southern blot hybridization, and the level of attenuation tested in a mouse model as described in the Examples below. Any deletion in the *rfbU* gene would result in the attenuation of the bacteria unless the deletion was small and allowed for functional *rfbU* expression. Any deletion which inactivates the *rfbU* gene expression or blocks function of its gene product will be both rough and attenuated.

For example, as described below in more detail, a deletion in the *rfbU* gene of *Brucella melitensis* was introduced by allelic exchange with a copy of the *rfbU* gene present on a vector. The *rfbU* gene to be exchanged with the wild type version contained a deletion into which a chloramphenicol acetyl transferase gene was cloned. Once the vector was introduced into *B. melitensis*, by electroporation in this example, a double cross-over recombinational event occured such that the vector *rfbU* gene containing the deletion, was exchanged for the chromosomal wild type *rfbU*. The resulting *B. melitensis* strain WRR51 contained a defined mutation was rough and attenuated, and did not possess resistance to an antibiotic used to treat brucellosis.

In another embodiment of the invention, the *Brucella* having the deletion in the *rfbU* gene as described above additionally contains another deletion in a different gene. The advantage of having two deletions is to further reduce the possibility of

reversion, and to additionally attenuate the bacteria. However, for use as a live vaccine a certain amount of replication is necessary in the host. Therefore, any vaccine strain designed in the methods of the present invention must be tested for its ability to survive in the host. These tests can be done *in vitro*, for example in a monocyte-derived macrophages system as described below in the Examples, or, as a second step, in non-human primates. It is preferable that the bacteria persists in the host for sufficient time to elicit a strong immunogenic response, for example from about four to six weeks. Bacteria too attenuated to survive enough to elicit an immunogenic response can be useful as diagnostic agents.

In the specific examples described below, a deletion in the *purE* gene of *B. melitensis* WRR51 was introduced using the allelic exchange procedure described above. The wild type *purE* locus was replaced with a deleted allele with a kanamycin resistance cassette inserted in the deletion site. The resultant double deletion strain (Δ*rfbU* Δ*purE*) was designated WRRP1. Other genes which can be used for introducing a second deletion include, but are not limited to e.g. *dnaKJ*, *recA*, genes potentially contributing to intracellular survival and proliferation (replication), for example by studying genes homologous to those found important in other intracellular bacterial pathogens or by screening for important genes for brucellar intracellular survival directly using IVET, or *in vivo* expression technology [Mahan, M. J. et al. (1993) *Science* **259**:686-688].

Both the single deletion strain, WRR51, and the double deletion strain, WRRP1, were tested in human-derived macrophages and were found able to infect human monocytes, and had reduced capacity to grow

within host cells. WRRP1, the double deletion, appeared to lose viability in host cells at a more rapid rate than those with a single deletion mutation such as WRR51, (Δ*rfbU*), or *B. melitensis* Δ*purE*. Thus, the subject strains should be able to persist in the host for extended periods of time, usually weeks, to enhance the effectiveness of the immunizing effect by continuous stimulation of the host immmune system until the host immune system has cleared all the organisms.

Ideally, for human administration, the vaccine strains should be sensitive to all antibiotics and synthetic antibacterials which are active against strains of *Brucella*. Even though strains containing these markers can be used as animal vaccines, it is preferable that strains selected on the basis of a selectable markers such as *cat* or kanamycin be further manipulated to remove these selectable markers. Methods for removing the marker gene include use of a "toxic" gene in vector as counterselectable marker to insure double crossover event e.g. *sacB*. These markers would be removed by a second allelic exchange, homologous recombination, this time with a copy of the gene containing the same deletion, but without the antibiotic resistance cassette inserted and verified by phenotype screening and Southern blot analysis. It is preferable to avoid providing resistance genes that can be disseminated through the environment or within the host to other pathogens.

The subject vaccines may be used in a wide variety of vertebrates. The subject vaccines will find particular use with mammals such as man and domestic animals. Domestic animals include bovine, ovine, porcine, equine, caprine, domestic fowl, Leporidate e.g., rabbits, or other animal which may be

held in captivity or may be a vector for a disease affecting a domestic animal such as a marine mammal.

The purified vaccine solution is prepared for administration to mammals by methods known in the art, which can include filtering to sterilize the solution, diluting the solution, adding an adjuvant and stabilizing the solution. The vaccine can be lyophilized to produce a vaccine against brucellae in a dried form for ease in transportation and storage. Further, the vaccine may be prepared in the form of a mixed vaccine which contains the deletion strains as described above and at least one other antigen as long as the added antigen does not interfere with the effectiveness of the vaccine and the side effects and adverse reactions are not increased additively or synergistically.

The vaccine may be stored in a sealed vial, ampule or the like. The present vaccine can generally be administered in the form of a liquid or suspension. In the case where the vaccine is in a dried form, the vaccine is dissolved or suspended in sterilized distilled water before administration. Generally, the vaccine may be administered orally, subcutaneously, intradermally or intramuscularly but preferably intranasally in a dose effective for the production of neutralizing antibody and protection from infection or disease. The manner of application of the vaccines may be varied widely, any of the conventional methods for administering a live vaccine being applicable. These include, orally, on a solid physiologically acceptable base, or in a physiologically acceptable dispersion. The dosage of the vaccine (number of bacteria, number of administrations) will depend on route of administration and will vary according to the species to be protected.

When providing a patient with live bacteria vaccines, the dosage of administered agent will vary depending upon such factors as the route of administration, patient's species, age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds which is in the range of from about 10 cfu/kg to 10^6 cfu/kg (body weight of patient), although a lower or higher dosage may be administered. For calves, for example, administration of live bacteria can be by intramuscular injection of by feeding in doses which are safe and may be appropriate. For example, at 10 months of age, heifers can be injected subcutaneously in the axillary area with 1-1.4 x 10^{10} cfu [Cheville, N. F. et al. (1993) *Am. J. Vet. Res.* **54**: 1591-1597]. One or more additional administrations may be provided as booster doses, usually at convenient intervals such as two to three weeks.

In another embodiment, the present invention related to a method and composition for delivering antigens or genes into cells. One or more of the desired antigens, or genes coding for these antigens, can be introduced into the live brucellae strains described above for use as a vaccine, and can be used only to provide said antigen, i.e. as a delivery vehicle, or to provide protection as a vaccine and deliver the desired antigen. The desired gene or antigen can be introduced into the bacteria either as episomal DNA, or as part of the *Brucella* chromosome by recombination for example, advantageously inserted in the deletion site of the vaccine strain, or replacing the selectable marker used in selecting the vaccine strain. Genes of interest may come from diverse

sources, such as bacteria, viruses, fungi, protozoa, metazoan parasites or the like. The structural genes may encode envelope proteins, capsid proteins, surface proteins, toxins, such as exotoxins or enterotoxins, or the genes of interest may specify proteins, enzymes, or oligosaccharide antigen or for modification of a saccharide-containing antigen, such as LPS, of the host bacterial strain, or for synthesis of a polypeptide antigen. Specific examples of genes of interest include HIV vif, malarial circumsporozoite protein, HBV core protein, and arboviral coat protein, to name a few. The construct or vector containing the gene of interest may be introduced into the host strain by any convenient means such as conjugation, transformation, transfection, transduction, etc. The *Brucella* containing the gene or antigen of interest is then allowed to enter the cell by infection, wherein the bacteria can replicate for a limited time thereby providing the antigen or gene of interest inside the cell. Additional administrations of the antigen or gene of interest can occur depending on the amount of antigen desired in the infected cell.

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

The following MATERIALS AND METHODS were used in the examples that follow.

Strains and growth conditions. All *Brucella* strains were grown in brucella broth or agar or M9 minimal agar. *B. melitensis* strains 16M (wild type),

ΔpurE201 [Drazek et al. (1995) *Infect. Immun.* **63**:3297-3301], WRR51 and WRRP1 were used to infect both monocyte-derived macrophages (MDMs) and mice. Stocks of exponential-phase cultures frozen in 20% glycerol were thawed and used to inoculate. Broth cultures were shaken at 37°C for 22 to 26 hours until an A_{600} of 0.4 to 0.6 was obtained. Bacterial cells were then harvested by centrifuge, washed in sterile 0.9% NaCl, and adjusted to the correct dilution in the same. Turbidity was measured by spectrophotometer (Spectronic 20, Spectronic Instruments, Inc., Rochester, NY) to estimate viable bacterial counts and thus determine the appropriate dose for infection. Actual colony-forming units (CFUs) in the inocula were determined by serial dilution and plating on *Brucella* agar. Plate counts were made after four days of incubation at 37°C, unless otherwise noted.

Animals. BALB/C female mice were obtained from Harlan Sprague Dawley,Frederick, MD, and were used at eight to twelve weeks of age. Mice were *Brucella*-free and were kept in a Biosafety level three (BSL-3) facility. Studies were performed according to Armed Forces Institute of Pathology Regulation 70-1 and all regulations and guidelines regarding the use of laboratory animals in research.

Construction of B. melitensis rough mutants. The DNA sequence of pJM63 [Winter,A. J. et al. (1996) *Amer. J. Vet. Res.* **57**:677-683] flanking the Tn*5* insertion in the putative *rfbU* gene of *B. abortus* 2303 was obtained. The *rfbU* gene was subcloned, and two *Cla*I sites were used to delete an internal portion of the gene (between positions 1063 and 1670) containing

the Tn5 insertion, to create pJM83ΔClaI. A chloramphenicol resistance cassette was then place into the ClaI deletion site to make the mutator plasmid pRFBU1. *Brucella melitensis* 16M was grown for 20 hours in YENB [Sharma, R. C., and R. T. Schimke (1996) *BioTechniques* **20**:42-44], pelleted, washed in 1/2 volume cold 10% glycerol and resuspended in 1/10 volume of the same. In cuvettes 1μg of plasmid DNA was added to 100μl of the electrocompetent bacteria and then electroporated at 2.5 kV, 25 μF and 600 Ω. One ml of SOC (Life Technologies, Inc., Gaithersburg, MD) was added and the mix was incubated at 37°C with shaking for one hour. The electroporation mix was then plated on brucella agar containing 10 μg chloramphenicol per ml.

Chloramphenicol-resistant electroporants were then screened on brucella agar plates containing 100μg/ml ampicillin and on plates with 5μg/ml chloramphenicol and 50μg/ml ampicillin. Ampicillin-sensitive, chloramphenicol-resistant colonies were then tested for changes in LPS first by staining with crystal violet and then by agglutination with anti-brucellar LPS serum. The chromosomes of these transformants were isolated [Marmur, J. (1961) *J. Mol. Biol.* **3**:208-218] and examined by Southern hybridization. Chromosomal DNA preparations were digested with *EcoR*I and *Cla*I, electroporated on a 1% agarose slab gel in TAE buffer, and transferred in a positive pressure cell (Stratagene, LaJolla, CA) to a Nytran membrane (Schleicher and Schuell, Keene, NH). An *rfbU* probe was made by DNA amplification via the polymerase chain reaction (PCR) using oligonucleotide primers with the nucleotide sequences 5'-GGATGTCGACCCAGCCCTCCACATCAATAGC-3'(SEQ ID NO:3) and

5'-TTGCGGATCCTTTACTCGTCCGTCTCTTAC-3' (SEQ ID NO:4). *B. melitensis* 16M DNA served as template. The amplicon contained the *rfbU* gene, 606 bp of the upstream flank and 278 bp of the downstream flank. The probe was labeled nonisotopically (Amersham Life Sciences, Birminghamshire, England) and hybridised at 42°C.

Genetic complementation was used to confirm that the Δ*rfbU* 5.1 mutation caused the LPS defect in WRR51. The amplicon described above that contained an intact copy of *rfbU* was cloned into pBBR1MCS and the resultant construct was electroporated into WRR51. Presence of the plasmid in electroporants was verified by antibiotic selection and by plasmid isolation and analysis. WRR51 electroporants bearing the *rfbU* plasmid were tested for restoration of wild type LPS synthesis by crystal violet staining and by anti-LPS agglutination.

B. melitensis strain WRRP1 was constructed by electroporating WRR51 with pURE198 [Drazek et al. (1995) *Infect. Immun.* **63**:3297-3301]. Electroporants with resistance to kanamycin (50μg/ml) and chloramphenicol (5μg/ml) were selected on brucella agar plates and then replica plated on brucella agar containing 100μg/ml ampicillin, M9 minimal media agar and M9 agar with added purines (5 mM adenine, 0.3 mM guanine). Recombinational replacement of the *purE* locus with Δ*purE*198 was confirmed by Southern hybridisation. Chromosomal preparations were digested with *EcoRI* and *Hind*III and probed with a PCR amplification product that included most of the *purEK* operon (GenBank locus BMU10241) and was made using the oligonucleotide primers 5'-CACCATGCAGCCGACACA-3' (SEQ ID NO:5) and 5'-CCGCGCCGCAGATTCAGG-3' (SEQ ID NO:6).

Maintainance of the Δ*rfbU*5.1 mutation in WRRP1 was also verified by Southern blot.

Procedures not specified above were done by standard methodology [Ausubel et al. (ed.) 1994. Current protocols in molecular biology. Greene Publishing Associates, New York; and Sambrook et al. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, NY].

Human MDM infection. Mononuclear cells obtained by centrifuging leukopaks from normal donors over lymphocyte separation medium (Organon Teknika, Durham, NC). Counterflow centrifugal elutriation was then used to further purify the monocytes [L. M. Wahl et al. (1984) *Cell. Immunol.* **85**:373-383], resulting in preparations with greater than 95% viability (estimated by trypan exclusion) and <10% lymphocytes. Monocytes were suspended in MDM medium [RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) with 10% heat-inactivated human serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine and 10 ng/ml of recombinant human macrophage colony stimulating factor (Jay Stoudemire, Genetics Institute, Boston, MA)]. This suspension was added to wells in a sterile tissue culture plate and 10^5 cells per well were cultured as adherent monolayers at 37°C in a 5% CO_2 incubator. On the fourth day and again on the seventh day, half of the medium was removed and replaced with fresh medium. On the eighth day of the experiment, the media in the MDM culture wells was removed and replaced with the medium described above, except with 10% unheated normal serum in place of Sigma serum. Brucella strains were grown as stated above. Broth media did not contain antibiotics to avoid carryover; cultures

used to infect were plated selective and nonselective agar to verify that antibiotic resistance was maintained uniformly in strains which bear these markers. After the saline wash, brucellae were resuspended to 2 X 10^8 CFU/ml in saline and added to MDM wells at a multiplicity of infection of 10:1. Infections proceeded for 60 min. at 37°C, then monolayers were washed three times with RPMI with 10% Sigma serum and 2 mM L-glutamine. MDM medium with 1 µg/ml gentamycin was then added to wells and plates were incubated at 37°C in a 5% CO_2 incubator. At various timepoints plates were removed and MDM monolayers were washed as before, and then lysed by adding 0.1% Triton X-100 and mixing vigorously. Lysates were diluted serially in sterile saline, and these dilutions were plated on brucella agar to obtain viable counts. Presented here are mean values from three MDM wells, dilutions from each plated in duplicate. The data shown are representative of at least four different experiments.

Survival in mice. Groups of mice were inoculated intraperitoneally with various doses of *B. melitensis* 16M and of the mutants, or with 0.2 ml sterile saline in the case of the control group. Blood, spleens, liver and lungs were obtained from the infected and control mice at 3, 7, 14, 42, 56 and 84 days after infection. Three mice from each group were sampled at each timepoint. Organs were weighed and then homogenized with 1 ml sterile saline. Suspensions were then diluted in sterile saline and plated on brucella agar. Colonies of *Brucella* were counted after 4 days of incubation at 37°C, and then again after 6 days for slow-growing strains. The identity

of recovered colonies was confirmed by screening for antibiotic resistance and relevant phenotype.

EXAMPLE 1

The *B. abortus* gene containing the Tn*5* insertion was first cloned from the VTRA1 chromosome on an 11.6 kbp DNA fragment in the plasmid construct pJM63. From a number of subclones of the pJM63 insert, the nucleotide sequence of the 2693 bp region containing the transposon insertion was determined (SEQ. ID. NO: 1). The Tn*5* insertion was located within an open reading frame of 1233 bp which coded for a gene that was distantly related (40% amino acid similarity) to the sequence of the *Salmonella enterica LT2* RfbU. A deletion of 607 bp was made in the putative *rfbU* gene of pJM63 using *Cla*I restriction sites at nucleotide positions 1063 and 1670 in the DNA sequence. A 1053 bp cassette containing a chloramphenicol acetyl transferase gene (*cat*) was ligated into the deletion site to create the plasmid pRFBU1. pRFBU1 was electroporated into *B. melitensis* strain 16M and electroporants with pRFBU1 integrated were selected on *Brucella* agar containing chloramphenicol. Resistant colonies were then screened for the loss of ampicillin resistance, which indicated that the vector portion of pRFBU1 had been lost from the chromosome by a second crossover recombinational event. DNA hybridization confirmed that the chloramphenicol resistant and ampicillin sensitive electroporants had the deletion mutation carrying the chloramphenicol resistance cassette in place of the wild type chromosomal locus. The deletion strain, designated WRR51, was confirmed to be rough by staining with crystal violet and by lack of agglutination with an anti-LPS serum.

EXAMPLE 2

A *purE* deletion was then introduced into *B. melitensis* strain WRR51 by a similar allelic exchange procedure. The suicide vector pURE201 was used as previously described [Drazek, E. S. et al. (1995) *Infect. Immun.* **63**: 3297-3301] to replace the wild type *purE* locus with a deleted allele with a kanamycin resistance cassette inserted in the deletion site. The resultant double deletion strain (Δ*rfbU* Δ*purE*) was designated WRRP1.

EXAMPLE 3

Human monocyte-derived macrophages were infected with *B. melitensis* 16M, with *B. melitensis* Δ*purE* 201, and with rough mutants WRR51 and WRRP1 (Figure 1). The wild type parent strain increased over two logs in 72 hours, but the rough mutants decreased in count over the same timecourse. Counts of the rough mutant WRR51 decreased gradually, comparable with levels seen with *B. melitensis* Δ*purE* 201, while the rough *purE* strain, WRRP1, was reduced by a full log over 72 hours. These results indicated that though the rough mutants were fully able to infect human monocytes, they had a reduced capacity to grow within host cells. WRRP1 appeared to lose viability in host cells at a more rapid rate than WRR51 or *B. melitensis* Δ*purE* 201.

EXAMPLE 4

In the murine model, BALB/c mice were infected intraperitoneally with 10^5 brucellae and spleen counts were taken at timepoints over 12 weeks (Figure 2). Wild type *B. melitensis* 16M persisted in the spleens of infected mice for the full 12 weeks, increasing in count by a log in the first week and then gradually

decreasing by about a log over the 11 weeks that followed. Spleens of mice infected with double mutant WRRP1 were fully cleared of viable bacteria by one week. Rough mutant WRR51 and *B. melitensis ΔpurE* 201 persisted in the spleen at eight weeks, but were cleared by 12 weeks. While these strains were similar in the duration of survival in the mouse spleen, early counts of the *purE* mutant were two logs higher than those of WRR51. This result indicated that the rough mutants are reduced in their efficiency to infect mice, at least by the intrapertoneal route. Both *ΔpurE* 201 and WRR51 were attenuated relative to the wild type *B. melitensis*, but both were able to survive in mice for over eight weeks. The double deletion mutant appeared to be severely attenuated compared to either of the single deletion mutants, but recent experiments indicate that increased dosage of WRRP1 can extend its persistence in mice to beyond four weeks.

What is claimed is:

1. An isolated *rfbU* DNA fragment or any portion thereof.

2. An isolated and purified DNA fragment according to claim 1, wherein said fragment has the sequence of SEQ ID NO:1 or a portion thereof, or an allelic portion thereof.

3. An isolated and purified *rfbU* DNA fragment according to claim 2 which encodes 411 amino acids or a portion thereof.

4. The DNA fragment according to claim 3, wherein said DNA fragment encodes the amino acid sequence according to SEQ ID NO: 2 or a portion thereof.

5. A DNA fragment according to claim 3, comprising at least 30 nucleotides of the sequence set forth therein.

6. A recombinant DNA construct comprising:

(i) a vector, and

(ii) an isolated and purified *rfbU* DNA fragment.

7. A recombinant DNA construct according to claim 6, wherein said DNA fragment encodes 411 amino acids of RfbU.

8. A recombinant DNA construct according to claim 6, wherein said *rfbU* DNA contains a deletion.

9. A recombinant DNA construct according to claim 8 wherein said construct is pRFBU1.

10. A recombinant DNA construct according to claim 6, wherein said vector is an expression vector.

11. The recombinant DNA construct according to claim 7, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO: 2.

12. The recombinant DNA construct according to claim 6, wherein said vector is a prokaryotic vector.

13. A host cell transformed with a recombinant DNA construct comprising:

(i) a vector, and

(ii) a DNA fragment which encodes 411 amino acids of RfbU , or a portion thereof.

14. A host cell according to claim 13, wherein said cell is prokaryotic.

15. A host cell according to claim 14, wherein said cell is *Brucella*.

16. A host cell according to claim 15, wherein said *Brucella* is *Brucella melitensis*.

17. The host cell of claim 16 wherein said recombinant DNA construct is pRFBU1.

18. The host cell of claim 17 wherein said cell is WRR51.

19. A method for producing RfbU which comprises culturing the cells according to claim 15, under conditions such that said DNA fragment is expressed and said RfbU is thereby produced, and isolating said RfbU.

20. An antibody to a peptide having the amino acid sequence specified in SEQ ID NO:2, or any portion thereof.

21. A method for the diagnosis of brucellosis infections comprising the steps of:

(i) contacting a sample from an individual suspected of having a brucellosis infection with an antibody according to claim 20; and

(ii) detecting the presence or absence of brucellosis by detecting the presence or absence of a complex formed between the RfbU produced as a result of said brucellosis and antibodies specific therefor.

22. A method for the diagnosis of brucellosis infection comprising the steps of :

(i) contacting a sample from an individual suspected of having a brucellosis infection with at least one attenuated strain of *Brucella* and

(ii) detecting the presence or absence of a brucellosis infection by detecting the presence or absence of a complex formed between the attenuated *Brucella* and antibodies specific therefor in the sample.

23. A method for detecting *rfbU* in a sample using the polymerase chain reaction, said method comprising:

(i) extracting DNA from the sample;

(ii) contacting said DNA with

(a) at least four nucleotide triphosphates,

(b) a primer that hybridizes to *rfbU* DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

(iii) denaturing said duplex to release said first DNA product from said DNA;

(iv) contacting said first DNA product with a reaction mixture comprising:

(a) at least four nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

(v) denaturing said second DNA product from said first DNA product;

(vi) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

(vii) fractionating said first and second DNA products generated from said *rfbU* DNA; and

(viii) detecting said fractionated products for the presence or absence of *rfbU* in a sample.

24. A brucellosis infection diagnostic kit comprising an antibody according to claim 20 and ancillary reagents suitable for use in detecting the presence or absence of RfbU in a mammalian sample.

25. A *Brucella* infection diagnostic kit comprising primers specific for *rfbU* and ancillary reagents suitable for use in detecting the presence or absence of *rfbU* in a mammalian sample.

26. A live *Brucella* vaccine comprising a strain of *Brucella* wherein said strain contains a deletion in a *rfbU* gene.

27. A live *Brucella* vaccine according to claim 26 wherein said *Brucella* strain further contains a deletion in a *purE* gene.

28. A live *Brucella* vaccine according to claim 26 wherein said *Brucella* is *Brucella melitensis*.

29. A live *Brucella* vaccine according to claim 28 wherein said *Brucella* is WRR51.

30. A live *Brucella* vaccine according to claim 27 wherein said *Brucella* is *Brucella melitensis*.

31. A live *Brucella* vaccine according to claim 30 wherein said *Brucella* is WRRP1.

32. A multivalent live *Brucella* vaccine comprising different strains of *Brucella* wherein each of the strains contains at least one defined deletion.

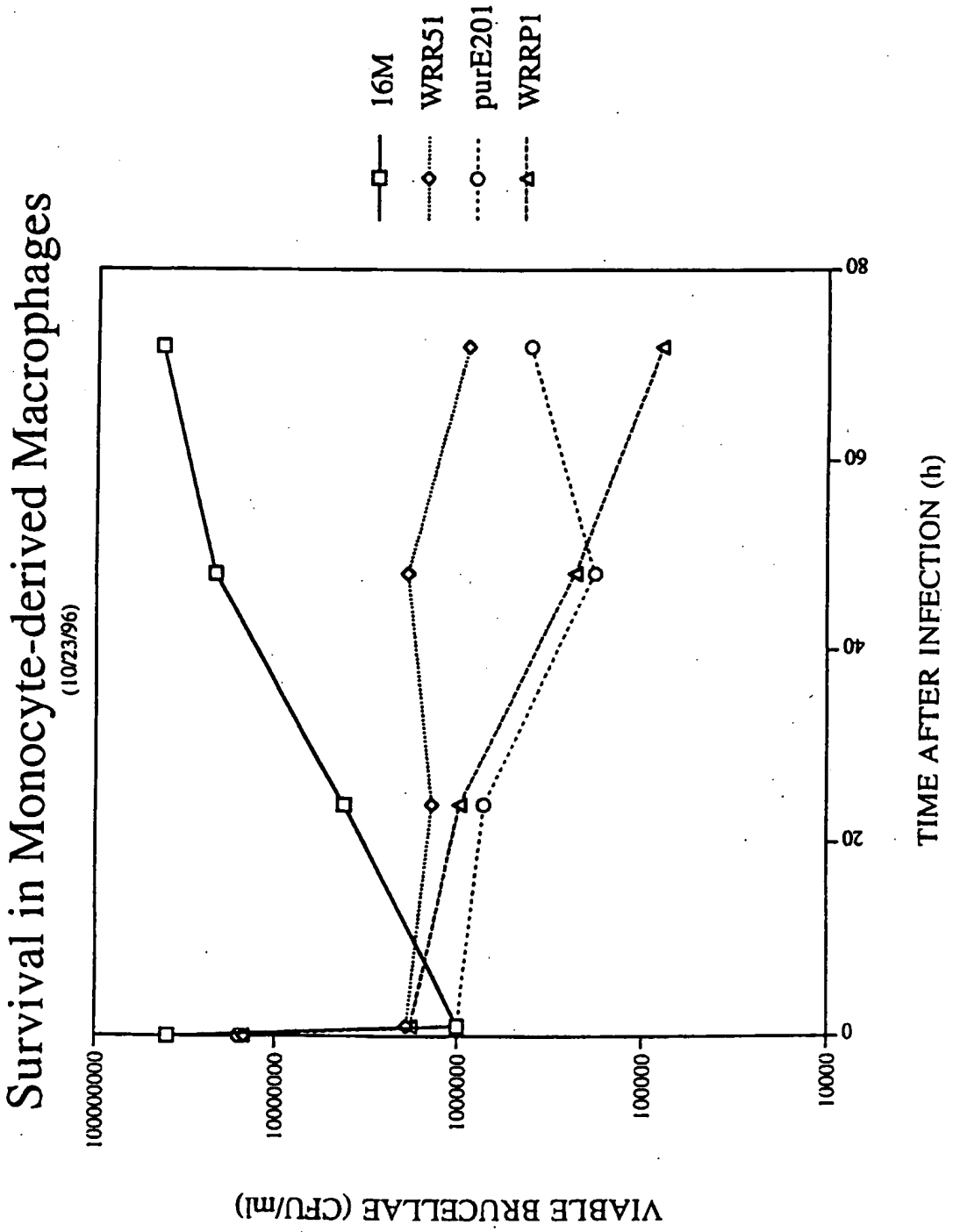
Survival in Monocyte-derived Macrophages
(10/23/96)
16M
WRR51
purE201
WRRP1
VIABLE BRUCELLAE (CFU/ml)
100000000
10000000
1000000
100000
10000
TIME AFTER INFECTION (h)
0
20
40
60
80

FIGURE 1

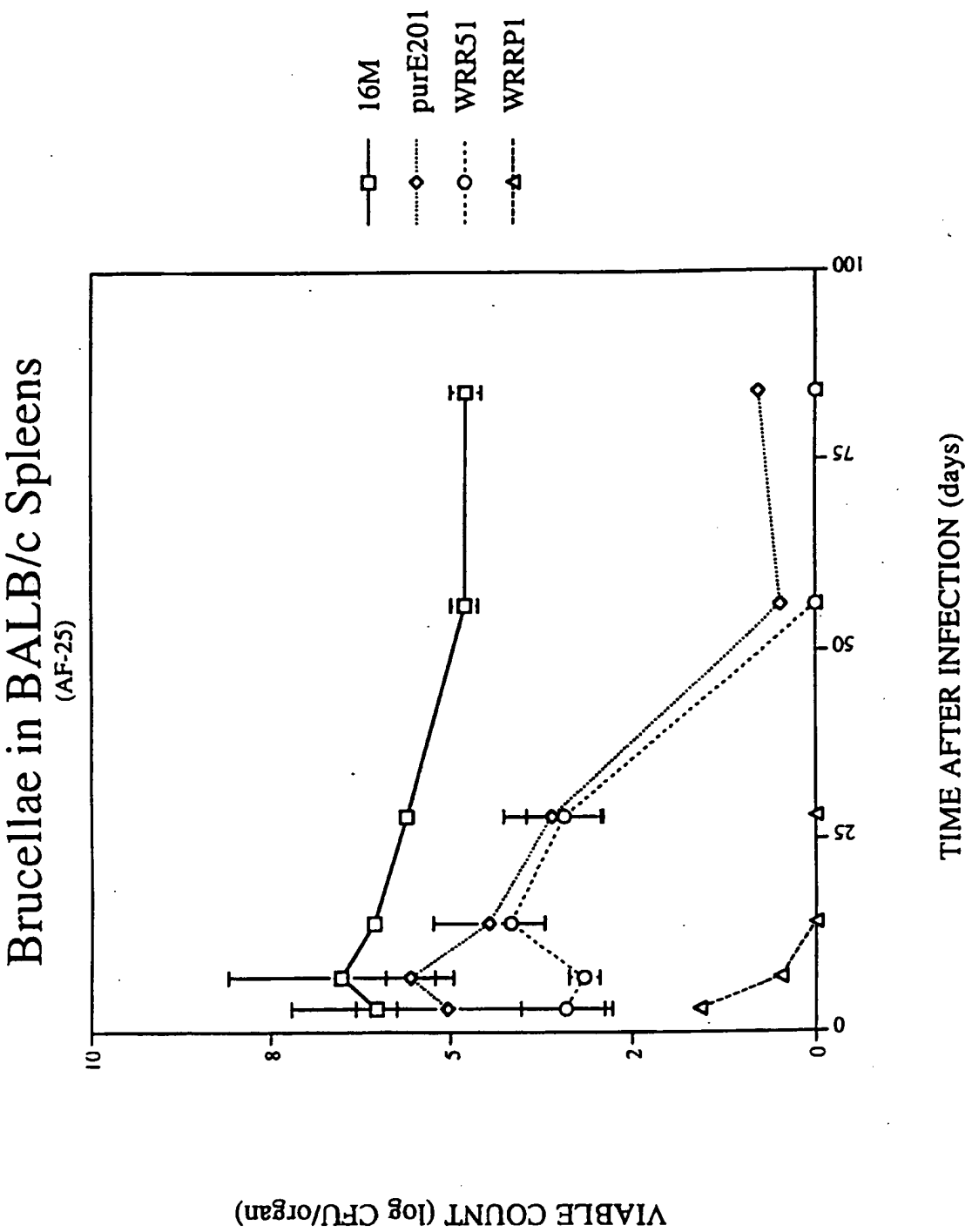
16M
purE201
WRR51
WRRP1
Brucellae in BALB/c Spleens
(AF-25)
VIABLE COUNT (log CFU/organ)
TIME AFTER INFECTION (days)
0
2
5
8
10
0
25
50
75
100

FIGURE 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Nikolich, Mikeljon
Hoover, David L.
Warren, Richard L.
Lindler, Luther E.
Hadfield, Ted L.
Boyle, Stephen M.
McQuiston, John R.
Schurig, Gerhardt G.
Nammalwar Sriranganathan

(ii) TITLE OF INVENTION: Live Vaccines Against Brucellosis

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Charles H. Harris
(B) STREET: 504 Scott Street, USA MRMC - MRMC-JA
(C) CITY: Fort Detrick
(D) STATE: MARYLAND
(E) COUNTRY: USA
(F) ZIP: 21702-5012

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.5
(D) SOFTWARE: Microsoft Word 6.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Harris, Charles H.
(B) REGISTRATION NUMBER: 34,616
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION
(A) TELEPHONE: (301) 619-2065
(B) TELEFAX: (301) 619-7714

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2693 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:Double
(D) TOPOLOGY: Linear

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACCTTATGT TTGGGACATT TTAATTAGGA ACGTTTATGC 40

CTTCGGATGC CGTGGGCGTG GCATCCGCAT GAGGGATGGC 80

TTTGCGTTTC TGCGCTTTGA AGATGTTGAA ATTGGGTTAG 120

GGCCGCAATA TGGTGGTGTA AGCCTACCAG CATATGAGTT 160

TCGAAATTTT GAGGGGTTAT TTCTTCGCCG CACCGAAGCC 200

ACTGGATTGG ATGGATATAC AGACCTTGGA TACGTCCCAG 240

ATGCTGAACA GCGGGGGTTC ATCTTTGCAG ACGGAGCAGC 280

CCTCCACATC AATAGCCTGT TTGCAGATAA TAACAAGGGT 320

GATGGCGTGT TTTGCCAAAA CGTCCAATAC GTAGATGGAA 360

ACGATCTCAA TTCATCCATC GACGGCGGAA CTGGGTTCAA 400

TTTTATCAAC GTAGATCGCA TAAACATCAA TACGATCCGC 440

AGTGGTGGCC GCCGGAATAT GGCACCAGGA AATCTTAACA 480

CTGTTTCCCA AGGTATCTCT TTGAATGCAA ATTGTCAGAC 520

TGTAATTATA GGCAACGCAG TTACCCACAA CTGGTGAAGT 560

CACGGTTTTT ATAGCCAAGC TCAGGACATT TTGGTTAATG 600

GTCTGATATC ACGTGATAAT GGCGGAAGGG GGTACGTTGC 640

AGAGGGTTCA GCAGGGTCAT CTCTCCTAAA TGGGGCCGTT 680

TTCAGAGATA ATGTAGCAGG GAATTATTTT ACAGGAGGGA 720

CAAGCGTAAA CCATCTCGCG AACCTCCAAC TTCATAACTC 760

TAGCACCGGG GGGAAAACTT TTGTGGCCAA TGTCACCACA 800

AATGGGTCTG CATAACGGTC CTTGCCATTT TAACTATAAA 840

TGAGCTATTC CCGCGCATTA AGAGTAGACA CGGGAAATCA 880

GTATGGCTCC GAGACATATT ACAGTTATCC TACCAGTTAA 920

GTACCGAGGC GGAAGTCTTC GAGTTACGAA GAATATCGTT 960

CGAATGCTTT TGAAGGGAAG TCAGAATTAT GGTGAACAGT 1000

GTCAAGTTAG ATTGGCAGTA CGTGCCGATA CCTACGATAT 1040

TGGGGAGGAG TTTCGTGATC TTATCGATAA TGGTGTAGAG 1080

GTTCGGGAAA TATCATTCAA AGAAGTTCCT CCAGAAGATG 1120

TTAACAATGC TAACTATTTC CAAGGTAGAA ATATCGACCT 1160

ACAGTCGAGA ACCTATTGGC TAATGGAGGA TGGCCAAAAC 1200

AACTGTGCCG ATAGTGACCT TTGGCTAGTT GTATCCTACT 1240

CTGTAGAGTA TCCTATTGCC CCAATAAGGC CGACTCTGAT 1280

ATTTGCCACC GATTTCATTC AAAGGTACGT ACCTGATATT 1320

ATTTGGCCAC CACGGCCCGG TGAGGGGGAT GCTGAGGCTC 1360

TTGCGTTCTT ACGACAATCA GACGGCGTAC TAGCTACAAC 1400

ACCACACACG CGGCTGGATG CGATTTCATA CGCTGGCTTA 1440

CCTGCGTCCA AAGTTTATCT TGCTCCGATG GAGTTTGACC 1480

CGACGTTTTT GGATCGTTAC CGGTCAGTGT CTAAGGTTAA 1520

GGAACCCTAT TTCCTTTGGC CAACCAACCC AAATGCTCAC 1560

AAAAACCATG CAAAAGCGTT TCAAGCGCTA GACCTATATT 1600

ACGGCAAACT AAAGGGTAAG ATAAAGACAA AGATAGTCGG 1640

TGTGAGTAGT GTGCGGATGG ACCCATCCCA TCGATGGCAG 1680

GCCAAGTACG AAAATAAGGC TTATGTGAAA TCTGTACGGG 1720

AAATTGTTGC GGGTCTCGAC AACCTGAAAA GCAATGTTGA 1760

GTTCGCTGGT GAGGTTGCGG ACAAGGAGTA TGCGGAGCTT 1800

CTTGCTTCAG CTTGTTTCTT TTGGCATCCA ACTTTGGCAG 1840

ACAACGGAAC TTTTGCTGCG GTCGAAGCAG CATATATGGG 1880

ATGTCCAACG CTTTCAAACG ACTACCCGCA GATGCGGTAT 1920

ATTTCTAACC GTTTCGAAAT TCCCATGCAG TATTTTAACG 1960

CAAGGTCTGT GAAGGAAATG GCATCAGCGC TTAAGCAAAT 2000

GGAGGAGACG CCAATAGATG TAGGTTTATT GCCAAGTCGA 2040

GAAACCCTAT CTCTGCATTC GTGGGAAGCT CACGCTTCCG 2080

AATACTGGGA TGTGATCGTG AGGGCAGCGG CATGAATAAG 2120

CTCGGCGTGT TTATCGGCTA TAACCCAGGC CAATTAGATC 2160

CATATCAGGG TATTTCTCGC TTAATTGCAT TCGTGATCAA 2200

GGGGGCCTTG AACCAGGGTA GCGGTGTAAC AATTGCTTGC 2240

CCCGGCTGGC TAAAGGACGA TGTACGTGTT CTTTGGGAAG 2280

ATGCTGATAT CCCACTTGAA GCGGTCAAAA TTATCGCGAC 2320

GAATGGTCAG CCTCCATTGG CTTCGTTATG GAAGTTGAGA 2360

GATAAGTTCC GTAAGAGACG GACGAGTAAA CGAAAACGTC 2400

TCTGGCTGGA GCGCTATGGA AAAAATGTTG CAAATTTTGT 2440

TGCAGAATGG CTTTCTTCGC GCTCGTATTG GGGGATTTTT 2480

TTGGGGGCTG CTGCAATTGC TGTAGTGACT ATTCTACTTG 2520

CCGTACCAAT TGCTATAGCC TTCACCGCTC TTATCGGCCT 2560

TCTATTTGCT CGTCGGCTTA TTAGACGTGT TATCAGGTCA 2600

AAGCTTGGTT TGTTTTTTCA CAAAAATGCC AATCAATTCA 2640

ACAAATTAAT GTCATCTGAT GAAACCATCG ACCGGATGAG 2680

GGAACGGGAA TTC 2693

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411
(B) TYPE: amino acids
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Pro Arg His Ile Thr Val Ile Leu
5 10

Pro Val Lys Tyr Arg Gly Gly Ser Leu Arg
15 20

Val Thr Lys Asn Ile Val Arg Met Leu Leu
25 30

Lys Gly Ser Gln Asn Tyr Gly Glu Gln Cys
35 40

Gln Val Arg Leu Ala Val Arg Ala Asp Thr
45 50

Tyr Asp Ile Gly Glu Glu Phe Arg Asp Leu
55 60

Ile Asp Asn Gly Val Glu Val Arg Glu Ile
65 70

Ser Phe Lys Glu Val Pro Pro Glu Asp Val
75 80

Asn Asn Ala Asn Tyr Phe Gln Gly Arg Asn
85 90

Ile Asp Leu Gln Ser Arg Thr Tyr Trp Leu
95 100

Met Glu Asp Gly Gln Asn Asn Cys Ala Asp
105 110

Ser Asp Leu Trp Leu Val Val Ser Tyr Ser
115 120

Val Glu Tyr Pro Ile Ala Pro Ile Arg Pro
125 130

Thr Leu Ile Phe Ala Thr Asp Phe Ile Gln
135 140

Arg Tyr Val Pro Asp Ile Ile Trp Pro Pro
145 150

Arg Pro Gly Glu Gly Asp Ala Glu Ala Leu
155 160

Ala Phe Leu Arg Gln Ser Asp Gly Val Leu
165 170

Ala Thr Thr Pro His Thr Arg Leu Asp Ala
175 180

Ile Ser Tyr Ala Gly Leu Pro Ala Ser Lys
185 190

Val Tyr Leu Ala Pro Met Glu Phe Asp Pro
195 200

Thr Phe Leu Asp Arg Tyr Arg Ser Val Ser
205 210

Lys Val Lys Glu Pro Tyr Phe Leu Trp Pro
215 220

Thr Asn Pro Asn Ala His Lys Asn His Ala
225 230

Lys Ala Phe Gln Ala Leu Asp Leu Tyr Tyr
235 240

Gly Lys Leu Lys Gly Lys Ile Lys Thr Lys
245 250

Ile Val Gly Val Ser Ser Val Arg Met Asp
255 260

Pro Ser His Arg Trp Gln Ala Lys Tyr Glu
265 270

Asn Lys Ala Tyr Val Lys Ser Val Arg Glu
275 280

Ile Val Ala Gly Leu Asp Asn Leu Lys Ser
285 290

Asn Val Glu Phe Ala Gly Glu Val Ala Asp
295 300

Lys Glu Tyr Ala Glu Leu Leu Ala Ser Ala
305 310

Cys Phe Phe Trp His Pro Thr Leu Ala Asp
315 320

Asn Gly Thr Phe Ala Ala Val Glu Ala Ala
325 330

Tyr Met Gly Cys Pro Thr Leu Ser Asn Asp
335 340

Tyr Pro Gln Met Arg Tyr Ile Ser Asn Arg
345 350

Phe Glu Ile Pro Met Gln Tyr Phe Asn Ala
355 360

Arg Ser Val Lys Glu Met Ala Ser Ala Leu
365 370

Lys Gln Met Glu Glu Thr Pro Ile Asp Val
375 380

Gly Leu Leu Pro Ser Arg glu Thr Leu Ser
385 390

Leu His Ser Trp Glu Ala His Ala Ser Glu
395 400

Tyr Trp Asp Val Ile Val Arg Ala Ala Ala
405 410

OPA

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGATGTCGAC CCAGCCCTCC ACATCAATAG C 31

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGCGGATCC TTTACTCGTC CGTCTCTTAC 30

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CACCATGCAG CCGACACA 18

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS:Double
(D) TOPOLOGY: Linear

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGCGCCGCA GATTCAGG 18